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Multi-class analysis of 46 antimicrobial drug residues in pond water using UHPLC-Orbitrap-HRMS and application to freshwater ponds in Flanders, Belgium

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ABSTRACT

Increasing anthropogenic pressure and agricultural pollution raises concerns regarding antimicrobial resistance and biodiversity loss in aquatic environments. In order to protect and restore water resources and biodiversity, antimicrobial drug residues should be monitored in all aquatic environments including pond water. Consequently, the objective of this research was to develop and validate a novel multi-residue method for the simultaneous quantification of 46 targeted human and veterinary antimicrobial drugs in pond water. A suitable extraction method based on solid-phase extraction (SPE) was developed, assisted by a fractional factorial design. A broad polarity range of compounds was covered (log P from −4.05 to 4.38), including major representatives of the following classes: sulfonamides, tetracyclines, quinolones, macrolides, lincosamides, nitrofurans, penicillins, cephalosporins, diaminopyrimidines, pleuromutilins and phenicols. All analytes were separated using ultra-high performance liquid chromatography (UHPLC) and detected in full-scan by Orbitrap high resolution mass spectrometry (Orbitrap-HRMS). Good linearity was obtained for all compounds with $R^2 \geq 0.993$ and goodness-of-fit coefficient (g) $\leq 11.56\%$. Method detection limits ranged from 10 to 50 ng L^{-1} and method quantification limits were 50 ng L^{-1} for all compounds. Acceptable values were obtained for within-day and between-day apparent recoveries (i.e. between 50 and 120%), precision (< 30% and < 45%) and measurement uncertainty (< 50%). Targeted analysis of 18 freshwater ponds throughout Flanders was performed to demonstrate the applicability of the newly developed UHPLC-HRMS method. Overall, 20 antimicrobial drugs were detected with highest concentrations observed for tetracyclines and their transformation products ranging between 51 and 248 ng L^{-1}. Finally, suspect screening was performed suggesting the presence of 14 additional pharmaceuticals including 3 antimicrobial degradation products (e.g. apo-oxytetracycline, amoxicillin penicilloic acid and penilloic acid) and 11 pesticides.

1. Introduction

The occurrence of trace levels (ng L^{-1} to μg L^{-1}) of antimicrobial drug residues (ADRs) in the aquatic environment is raising concern due to the potential link to increased resistance in natural bacterial populations [1]. Moreover, several ADRs alter the growth of aquatic microorganisms that are important for the ecological balance of the food chain and the conservation of biodiversity. For example, in the case of lincomycin, florphenicol, tetracycline, oxy- and chlortetracycline, toxic effects were observed in algae (i.e. *Pseudokirchneriella subcapitata*) and

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crustaceans (i.e. *Ceriodaphnia silvestrii*) with a half maximal effective concentration (EC_{50}) of less than 1 mg L^{-1} in the aquatic environment [2,3]. Furthermore, increasing anthropogenic pressure and agricultural pollution is affecting disease dynamics of emerging fungal infectious diseases such as chytridiomycosis in amphibians and this either directly (i.e. increasing disease susceptibility of the amphibian) [4] or indirectly (i.e. reducing zooplankton as part of the amphibian diet) [5]. Studies have highlighted the importance of zooplankton as natural predators of chytrid spores and their ability of reducing infection rates [6]. Since chytridiomycosis is causing major population declines of amphibian species and because these species are a crucial element in aquatic food webs, their loss induces drastic changes in ecosystem functioning and its structure [6]. Understanding environmental factors that influence chytridiomycosis, e.g. contamination status in ponds, can provide unprecedented opportunities for disease mitigation by biomanipulating the situation towards pathogen endemism, e.g. host-pathogen co-existence [6]. In light of growing concerns regarding antimicrobial resistance and toxicity and because ponds play key role in amphibian biodiversity by forming a habitat for amphibians, zooplankton and chytrid fungi, it is highly recommended to determine ADRs in pond water as was previously stated by Oertli et al. (2005) [7] and Rollins-Smith et al., 2011 [4].

The most significant entry route of human antimicrobial drugs to the aquatic environment concerns their incomplete removal at sewage treatment plants [1]. In contrast, most veterinary antimicrobial drugs find their way into the aquatic environment by application of liquid manure on fields during agriculture and subsequent mechanisms of surface run-off, leaching and drift, the latter being displacement of aerosolized manure droplets after certain manure spraying techniques. Even after metabolization in the animal body and differential sorption to sludge and soil, several antimicrobial drugs are found in surface water bodies adjacent to agricultural fields at concentrations ranging from 3 to 4,880 ng L^{-1}, as has been reported for sulfamethoxazole (3 ng L^{-1}) in river water and for tylosine (39 ng L^{-1}) and tetracycline (4,880 ng L^{-1}) in ditches and ponds around agricultural farms [8,9].

Up to now, research has mainly focused on the detection of ADRs in groundwater, river water, drinking water and wastewater [10,11] and only very few studies have been dedicated to the detection of a limited number of ADRs ($n \leq 10$) in natural ponds [12]. However, comparing biodiversity of macrophytes and macroinvertebrates, Williams et al. (2003) [13] concluded that ponds at the regional level contributed most to biodiversity by supporting more unique species as do rivers, streams and ditches. For this reason and due to the concerns mentioned above, investigation into the prevalence of ADRs in pond water has been recommended as well [2,4,5]. Ponds are defined as waterbodies between 1 m^2 and 2 ha in area which may be permanent or seasonal with a maximum depth of no more than 8 m [7]. Several hurdles need to be overcome to enable the successful extraction and detection of ADRs in fresh pond water i.e. the presence of organic matter and overall matrix variability, which implies the need for appropriate filters and internal standards. In addition, high sensitivity to detect low concentrations (ng L^{-1} range) and formed metabolites and transformation or degradation products needs to be considered as well [14,15].

Most of the previous work reporting on ADRs in the aquatic environment focused on parent compounds from 2 to 10 different classes with quantification limits ranging between 0.1 and 100 ng per liter [10,16]. Very few papers have reported on the determination of antimicrobial transformation products in the aquatic environment [17,18] and to best of the authors' knowledge no research so far was performed on the determination of these compounds in pond water. It is however well documented that tetracyclines can undergo epimerization in the presence of water to 4-epitetracycline, 4-epichlortetracycline and 4-epioxytetracycline, the latter expressing lower antibiotic activity. However, under certain conditions epimerization products can convert back into the parent tetracycline [17]. This exemplifies why not only the concentration of tetracycline residues but also their transformation products, are important to monitor in understanding the potential effects of ADRs in the aquatic environment.

High-resolution MS (HRMS) combines sensitive full-spectrum data with high mass resolution and mass accuracy. Since acquisition is not targeted, any ionizable compound in the sample can be detected as is the case during suspect screening, without requiring a preselection of analytes or without having reference standards available [19]. Few studies have been performed using (UHP)LC-HRMS for the detection of antimicrobials in river water ($n \leq 8$) [20], wastewater ($n = 11$) [21] and drinking water ($n = 1$) [22]. To the authors' knowledge, this is the first study to combine targeted quantification of a large number of multi-class ADRs ($n = 46$) in pond water together with suspect screening using solid-phase extraction (SPE) coupled with UHPLC-HRMS.

2. Materials and methods

2.1. Chemicals and reagents

Forty-six antimicrobial drug analytical standards (Table S1) were purchased from Sigma-Aldrich (Overijse, Belgium), Clearsynth (Mumbai, India), Bioconnect (Huissen, The Netherlands) and Council of Europe (Strasbourg, France). A broad polarity range of compounds was covered (log P from −4.05 to 4.38), including major representatives of the following 11 classes: sulfonamides ($n = 8$), tetracyclines ($n = 7$), quinolones ($n = 11$), macrolides ($n = 3$), lincosamides ($n = 1$), nitrofurans ($n = 4$), penicillins ($n = 5$), cephalosporins ($n = 3$), diaminopyrimidines ($n = 1$), pleuromutilins ($n = 1$) and phenicols ($n = 2$). The selected internal standards for each class ($n = 11$) were purchased at Sigma-Aldrich (Overijse, Belgium) (Table S1). Primary stock solutions were prepared in methanol at a concentration of 1 mg mL^{-1}, except for penicillins which were prepared in Milli-Q Ultrapure water [23]. Standard work solutions were prepared in Milli-Q Ultrapure water, thereby reaching a concentration between 0.1 and 100 μg mL^{-1}. Stock and work solutions were stored in opaque cups at −20 °C except for solutions of cephalexin, cefquinome, penicillin G, penicillin V, amoxicillin-diketopiperazine-2′5′-dione and amoxicillin-$^{13}C_6$ which were stored in opaque cups at −80 °C. The organic solvents were of HPLC grade, purchased from Fisher Scientific (Loughborough, UK). Formic acid (98%) and ammonium solution (NH_4OH, 25%) for analysis were purchased from Merck (Darmstadt). Ethylenediaminetetraacetic acid (EDTA) was of AnalaR® grade from BDH (Poole, Dorset, UK). The C18 Oasis® Hydrophilic-Lipophilic-Balanced (HLB) cartridges (500 mg, 6 mL) were purchased from Waters (Zellik, Belgium).

2.2. Sample preparation and extraction

2.2.1. Statistical model for extraction optimization

The software program JMP 12.0 (SAS Institute Inc, Cary, USA) was used to fit, assess and model three statistical experimental designs including a screening design to develop the most efficient extraction method (24 experiments), an eluent optimization design (10 experiments) and a response surface modelling design (RSM) (10 experiments). For this purpose, tap water was used, spiked with a mixture of the 46 ADRs to reach 100 ng L^{-1}. One-sample t-tests at the confidence interval of 95% (t-ratio $> |1.96|$ and p-value < 0.05) were used to statistically evaluate responses generated by each statistical design. The t-ratio is the ratio of the difference between the mean of two sample sets (generated by two extraction parameters) and the variation that exists within the sample sets (i.e. the standard error). A t-ratio greater than 1.96 in absolute value indicates that groups are significantly different at the 95% confidence interval. Twelve parameters that could affect the ADRs extraction efficiency were selected based upon literature [17] and tested for effect during the screening phase. Selected parameters were related to sample treatment (i.e. EDTA addition, acidification and filtration of the sample), SPE (i.e. conditioning, equilibration, loading and wash volume, dry time and elution solvent, volume and modifier) and sample evaporation (i.e. evaporation temperature). Significance

was determined by using a three-level fractional factorial resolution IV experimental design. Parameters with a significant effect on the extraction, i.e. t-ratio greater than 1.96 in absolute value were retained for further optimization (Fig. 2). Secondly, the elution solvent was optimized using a simplex lattice mixture design for three variables (percentage of methanol, methyl *tert*-butyl ether and acetonitrile). Thirdly, the selected significant variables were optimized through RSM using a central composited faced-centered (CCF) design. Optimization was performed using the summarized normalized area considering the number of analytes detected while ensuring equal compound distribution [14].

2.2.2. Final sample preparation and extraction method

Grab water samples were collected from the water surface with plastic buckets without disturbing the sediment layer and filtered on spot to eliminate organic matter such as algae, water plants and leaves (Retsch® sieve, Novolab NV, Geraardsbergen, Belgium, 250 μm, 50 × 200 mm). Samples were poured in glass amber bottles rinsed with 1.0 M EDTA to prevent complexation of tetracyclines with Ca^{2+} and Mg^{2+} ions and residual metal ions [24]. Prior to extraction, 500 mL water samples were filtered for a second time to remove all particles able to plug up the SPE cartridges and hence slow down the sample preparation step (Glass Microfiber Filters Whatman™, GE Healthcare Life Sciences, Buckinghamshire, United Kingdom, 0.45 μm, 90 × 90 mm). A disposable C18 Oasis® Hydrophilic-Lipophilic-Balanced (HLB) cartridge (500 mg, 6 mL) was used for further clean up and concentrate samples (Waters, Zellik, Belgium). The cartridge was preconditioned with 3 mL of methanol and 7 mL of ultrapure water. Samples were loaded under vacuum. Next, the cartridge was washed with 5 mL of ultrapure water and dried under vacuum for 15 min. Analytes were eluted with 3.5 mL of methanol/acetonitrile/methyl *tert*-butyl ether (33%, v/v/v) acidified with 0.1% formic acid, following 3.5 mL of the same mixture alkalinized with 0.1% ammonia 25% solution. The eluate was evaporated until dryness under a gentle nitrogen stream at 45 °C in a water bath. Reconstitution was performed using 100 μL of methanol following 50 μL of ultrapure water resulting in a 150 μL extract corresponding to 500 mL of sample (i.e. a concentration factor of 3333). Finally, samples were centrifuged for 5 min at 2430×*g* and the clear supernatant was collected into LC-MS glass vials and injected subsequently.

2.3. Instrumentation

Chromatographic separation of ADRs was achieved using an Ultimate 3000 XRS UHPLC system (Dionex, Amsterdam, The Netherlands) equipped with a Hypersil Gold C18 100 × 2.1 mm UHPLC column (1.9 μm particle size) (Thermo Fisher Scientific, Merelbeke, Belgium) operating at 40 °C. The injection volume was 2 μL. The mobile phase consisted of a mixture of Milli-Q Ultrapure water (mobile phase A) and methanol (mobile phase B) both containing 0.1% formic acid (FA), pumped at a flow rate of 0.40 mL min-1. The linear gradient program was as follows: 0.0–1.0 min, 15% B; 1.0–7.0 min: linear gradient to 100% B; 7.0–9.0 min: 100% B; 9.0–9.1 min: linear gradient to 15% B; 9.1–11.0 min: 15% B. Tested solvent gradients are presented in supplementary data (Tables S2–S3).

The detection of ADRs was carried out using a Q-Exactive™ benchtop HRMS (Thermo Fisher Scientific, San Francisco, USA), equipped with a heated electrospray ionization (HESI) source. Nitrogen sheath gas, auxiliary gas and sweep gas flow were set at 35, 10 and 2 a. u. (arbitrary units), respectively. The capillary temperature was 350 °C, the spray voltage 2 kV or −2 kV in positive or negative ion modes, respectively. The optimal MS parameters were S-lens RF-level 70, full-scan events and operated in polarity switching mode. Scans were performed with a resolution of 140,000 FWHM (Full Width at Half Maximum) at a rate of 1.5 Hz and scan ranges from 100 to 1500 Da. The automatic gain control (AGC) was set at 1×10^5 and the maximum injection time was set at 50 ms. The instrument was calibrated by infusing calibration mixtures for positive and negative ion mode (LTQ Velos ESI positive and negative ion calibration solution, Thermo Fisher Scientific). Detection was based on calculated exact mass and retention time of target compounds, presented in Table S1. The Xcalibur 4.0 software (Thermo Fisher Scientific) was used for instrument control and data processing. The mass spectrometer was calibrated daily with a solution of caffeine, MRFA peptide, Ultramark 1621 and n-butyl-amine (Pierce™ LTQ Velos ESI Positive Ion Calibration Solution) according to the manufacturer's instructions using Tune instrument control software version 2.9.

2.4. Method validation

The optimized analytical method was validated using fresh tap water as no fully blank pond water samples were available containing no traces of antibiotics. However, possible matrix effects were corrected by using appropriate (isotopically labeled) internal standards. At present no specific validation guidelines are available for the analysis of micro pollutants in fresh (as well as salt) water ecosystems [14]. CD 2009/90/EC [25] is the only European guideline that is currently available for chemical evaluation of the water status. It states that the measurement uncertainty must be below 50% and the limit of detection has to be 30% below the environmental quality standard (EQS), with EQS defined as the concentration of a substance in water that should not be exceeded in order to maintain the environmental quality objective. However, currently no environmental quality standards have been listed in case of ADRs in the aquatic environment. For this reason, additional guidelines were consulted for additional performance criteria as to ensure full analytical method validation, i.e. CD 2002/657/EC [26], VICH [27] and ICH guidelines [28].

The performance of the method was assessed in terms of linearity, limit of detection (LOD) and limit of quantification (LOQ), within-day and between-day precision and apparent recovery, measurement uncertainty, carry-over and specificity. A threefold 7-point calibration curve (0, 50, 100, 200, 300, 400, 500 ng L^{-1}) was obtained. Linearity was evaluated on three different days using the coefficient of determination (R^2) and confirmed by the goodness-of-fit coefficient (g) (%), the latter covering the difference between nominal calibration curve value and calculated concentration (Eq. (1)). Acceptance criteria of R^2 and g were set at ≥ 0.99 and ≤ 20% [29].

$$g = \sqrt{\sum (\%deviation)^2} \Big/ (n-1)) \quad (1)$$

The LOD was determined as 3 times the standard deviation of the y intercept divided by the average slope of three individual calibration curves within a linearity range of 50–500 ng L^{-1} [27]. Additionally, blank samples were spiked at LOD level for each target compound and identity was confirmed through the use of ^{13}C-isotope and a stable $^{13}C/^{12}C$-ratio at the corresponding LOD [30]. The LOQ was determined by analyzing the lowest concentration at which the method was validated within the limits of apparent recovery and precision according to the guidelines described above. Apparent recovery values (R_{app}, %) were evaluated by analyzing 6 samples spiked at 50 ng L^{-1} (LOQ), 200 ng L^{-1} and 400 ng L^{-1}. R_{app} is defined as the ratio between the measured concentration and the theoretical (spiked) concentration (Eq. (2)). The acceptance criteria for within-day and between-day R_{app} were between 50% and 120% [26].

$$R_{app} = \frac{measured\ concentration}{theoretical\ concentration}\ x\ 100 \quad (2)$$

Furthermore, the within-day precision (repeatability) and between-day precision (reproducibility) were determined by calculating the relative standard deviation (RSD, %) of spiked concentrations at 1.0, 4.0 and 8.0 times the LOQ-level in six-fold. This procedure was repeated during three different days by two different operators. RSD values had to be lower than the maximum relative standard deviation (RSD_{max}, %)

which was < 30% for the within-day precision and < 45% for the between-day precision at concentrations < 1 ng mL^{-1} [27]. The measurement uncertainty (U) was determined by multiplying the combined standard uncertainty (u_c) with a coverage factor k = 2, providing a level of confidence of approximately 95%. The u_c equals the positive square root of the between-day precision (RSD_R) and the bias of the analytical method, the latter comprising the uncertainty of the purity of the used standards ($U_{[Cref]}$), the accuracy of the bias (S_{bias}) (i.e. the difference between the mean measured value from the test results and the reference value or R_{app}), and the root mean square of the bias (RMS_{bias}) (Eq. (3)) [31].

$$U = 2\,x\,u_c = 2\,x\sqrt{(RSD_R{}^2 + U_{[Cref]}{}^2 + S_{bias}{}^2 + RMS_{bias}{}^2)} \quad (3)$$

with $RMS_{bias} = \sqrt{\frac{\sum (bias_i)^2}{n}}$

Carry-over was assessed by analyzing a mixture of MeOH/water (85/15; v/v) directly after every calibrator (50–500 ng L^{-1}). Finally, specificity was initially determined in tap water and afterwards confirmed by checking the absence of possible interfering chromatographic peaks within the retention time margin of 5% for all target compounds at their accurate mass in 3 pond samples being blank for the compound of interest [28].

2.5. Application to environmental samples

Initial information on pond localization in Belgium was provided by the database of the Pondscape project [32]. Next, a geographical selection was made, using Qgis 2.14 software, reducing all ponds to those present in Flanders. Finally, a field study was performed and permanent dried up ponds or ponds with < 1 m^2 in area or > 8 m in depth were excluded from the study. Site locations and coordinates of the 18 selected ponds are presented in Fig. 1 and Table S4 of supplementary material, respectively.

From the selected ponds, fresh water grab samples of 2.5 L each were collected from August until October 2018. Pond depth and area ranged between 0.15 and 1.5 m and 19.6 and 270.2 m^2, respectively. Pond shapes were evaluated to distinguish 3 types, i.e. rectangular, elliptic and circular ponds. Two metal rods connected by a wire were placed at shore, dividing ponds in two equal halves (Fig. S2). Surface water samples were taken across the wire at < 1 m, 1 m and > 1 m from shore. Finally, samples were poured together and filtered before transporting them on ice to minimize the degradation of thermolabile antimicrobial drugs such as β-lactam antibiotics and tetracyclines [10]. Upon arrival in the lab, samples were filtered a second time and stored at 4 °C for less than 24 h until analysis. Each sample was analyzed in triplicate. During sample analysis, quality control was assured by using internal standards, a fresh-made calibration curve and six matrix-matched quality control samples at low, medium and high concentrations (i.e. 50 (n = 2), 200 (n = 2) and 400 ng L^{-1} (n = 2), respectively).

2.6. Data analysis

2.6.1. Targeted analysis

Identification and quantification of target compounds in full-scan data was executed by XCalibur 4.0 software (Thermo Fisher Scientific). Single compound identification was realized by use of the accurate mass (m/z) of the pseudo-molecular mass ion (mass deviation limit < 3 ppm) [14], the $^{13}C/^{12}C$-isotope pattern and the retention time relative to that of the internal standard (deviation ≤ 2.5%) [33], all being investigated from the corresponding reference standard.

2.6.2. Suspect screening and confirmatory analysis

Suspect screening was performed using ToxFinder™ software

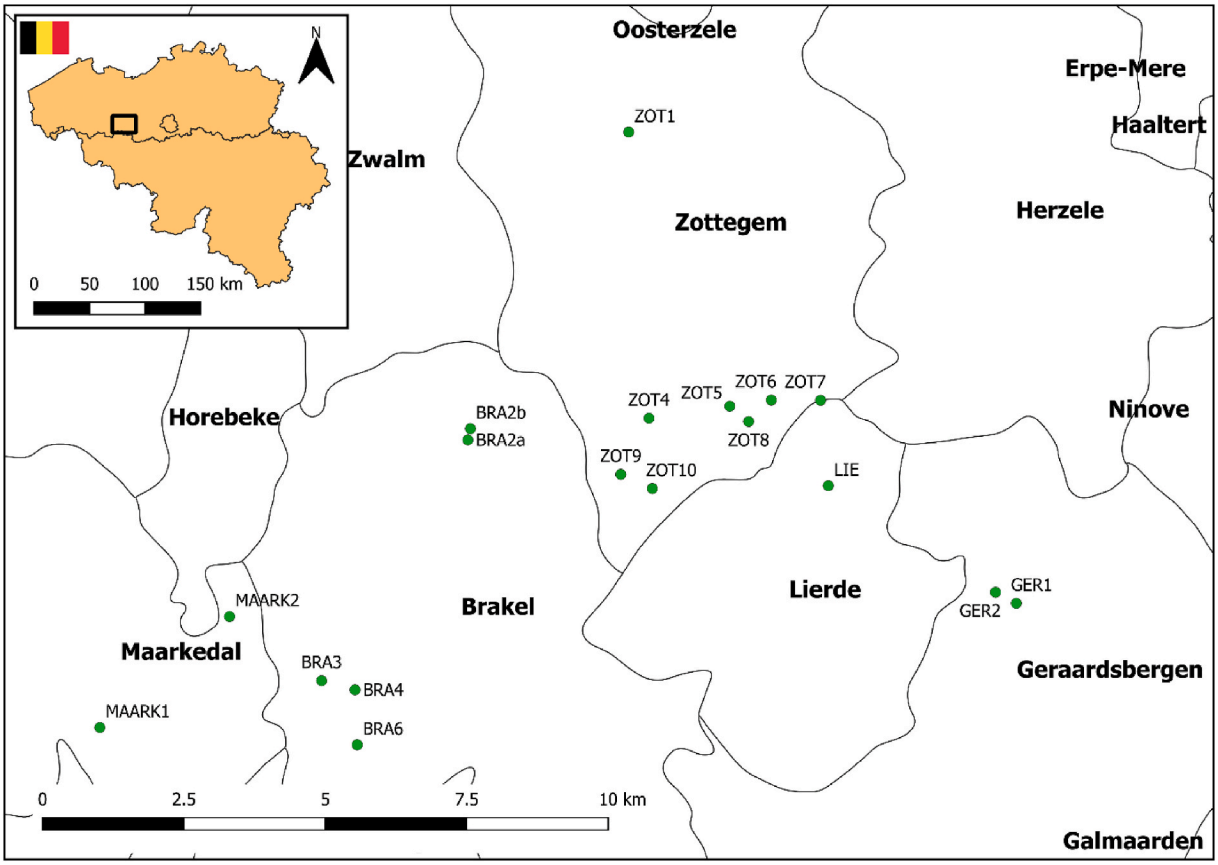

Fig. 1. Site locations and pond identification of the sampled freshwater ponds in Flanders, Belgium.

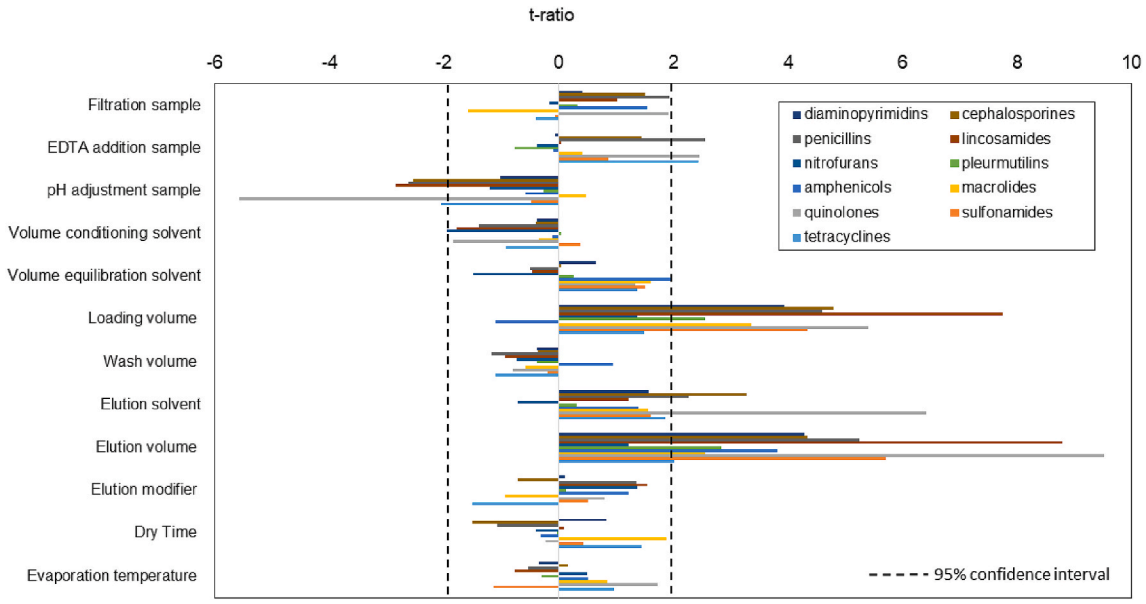

Fig. 2. T-ratio effect diagram, illustrating the significance of different extraction parameters for the 11 ADR classes. T-ratio effect bars crossing the 95% confidence interval (dashed line) indicate a significant effect of the respective parameter in the extraction process.

(Thermo Fisher Scientific, USA) in which chromatographic peak alignment and retrieving of components from full-scan data was performed within retention time limits of 0.5 min (lower limit) and 10 min (upper limit) for a scan range between 50 and 1000 m/z. An in-house database allowed screening of the full-scan HRMS spectra of each sample for the possible presence of regularly used pesticides (n = 83) (Table S6) and pharmaceuticals (n = 105) (Table S5), including other antimicrobial drugs than the 46 ADRs included in the target method (n = 17) and antimicrobial transformation products (n = 17) based on the *m/z* values. Inclusion criteria consisted of a minimum signal intensity of 100,000, a maximum mass deviation of 5 ppm and the presence of the ^{13}C-isotope. Blank samples (i.e. methanol) were used for subtraction of noise peaks in the samples of interest. A confirmatory analysis using purchased reference standards (Sigma-Aldrich, Overijse, Belgium) was performed to allow unequivocal compound identification. Proposed structures were confirmed through matching accurate mass (deviation limit < 3 ppm), RT time (deviation ≤ 2.5%) and the presence of the ^{13}C-isotope [34].

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

Oasis® HLB cartridges (6 mL, 500 mg, Waters) were selected based upon prior in-house testing and literature demonstrating the superiority of HLB cartridges to other cartridges (e.g. Oasis ® MCX) in the extraction of multi-class antimicrobials in surface water [11,21].

The extraction was further optimized using a three-level fractional resolution IV experimental design during which the effect of 12 parameters on the extraction efficiency of the 46 target ADRs was assessed. Seven parameters were non-significant, whereas the addition of EDTA, pH adjustment of the sample to 3, loading and elution volume as well as the type of elution solvent exerted a significant effect (t-ratio > $|1.96|$ and p-value < 0.05) on the summarized normalized area of more than one class of ADR. More specific, higher summarized normalized areas were observed with higher loading and elution volumes, acidification and addition of EDTA during sample preparation (Fig. 2). However, because β-lactams tend to degrade in acidic media due to the hydrolysis of the electrophilic β-lactam ring, samples were not acidified before extraction. Based upon these initial results and the different elution solvents described in literature [17,35], the elution solvent was further optimized using a simplex lattice mixture design. This resulted in an optimal mixture of 33% methanol, 33% acetonitrile and 33% methyl *tert*-butyl ether. Whereas in literature 100% organic solvents are frequently applied for the SPE elution of a maximum of 5 antimicrobial classes [9,11], this mixture offered an optimal compromise for the extraction of 46 ADRs from 11 different classes.

Finally, the loading volume and solvent additive were further optimized using RSM leading to higher loading volumes (> 200 mL) and different solvent additives for different ADR classes. However, loading volumes higher than 500 mL tended to clog the sorbent phase, resulting in long elution times, making the extraction method less practicable. For this reason, an optimal loading volume of 500 mL was selected. Optimization of the solvent additive demonstrated higher sensitivity for penicillins, nitrofurans, sulfonamides, lincomycins and amphenicols in the presence of 0.1% ammonium hydroxide whereas cephalosporins, quinolones, tetracyclines, aminoglycosides, macrolides and pleuromutilins showed higher recoveries in the presence of 0.1% formic acid. These findings are in line with literature, demonstrating different optimal solvent additives for different antimicrobial classes [10]. To establish an optimized extraction method for all antimicrobial classes, elution was performed using subsequently the acidified and the alkalinized solvent.

3.1.2. Liquid chromatography

Several authors have discussed on the superiority of LC to GC for the chromatographic separation of pharmaceuticals resulting from surface waters, as incomplete derivatization of polar compounds (e.g., atenolol, gentamicin) and degradation of thermolabile compounds (e.g., carbamazepine, amoxicillin) are major disadvantages of GC [36]. During the past decade, UHPLC has become the most important technique in LC. The UHPLC separation technique was selected in this study due to its higher performance in chromatographic resolution, shorter run times (<18 min) and increase in detection sensitivity, resulting from more narrow and sharp peaks compared to conventional HPLC [19]. By studying the symmetry of the peak shape ($As_{minimal}$ = 1.50 and $As_{optimal}$ = 1.00) and the resolution and interfering background of the 46 target ADRs, the optimal conditions of the stationary phase, flow rate, mobile phase composition and additives, solvent gradient, column temperature and injection volume were determined. Separation of 46 target ADRs

was achieved covering a broad polarity range (log P ranging from −4.05 to 4.38) with retention times ranging from 0.95 to 7.63 min (Table S1). Mass analogues (having overlapping mass extraction windows) were baseline separated as was the case for tetracycline, 4-epitetracycline and doxycycline with an accurate empirical mass of 445.16035 Da and 3 ppm mass tolerance. The inter-linked resolution (R_s) of different mass analogues was evaluated, considering a minimal resolution of 1.10 and an optimal resolution of 4.00. Chromatograms of each target ADR are presented in supplementary data (Fig. S1).

3.1.3. Mass spectrometry

The overall peak intensity of each ADR was the main evaluation criterion for the optimization of HESI ionization parameters (spray voltage, capillary voltage, tube lens voltage, skimmer voltage, heater and capillary temperature). The ADR mass spectra were characterized by the abundant presence of a positive or negative pseudo-molecular ion ($[M+H]^+$ or $[M-H]^-$ respectively) previously reported for antimicrobial drugs [10]. A resolving power of 140,000 FWHM was selected to ensure high mass accuracy and selectivity (mass deviation $\leq$ 1 ppm) (Table S1) and enable confident discrimination of coeluting, isobaric compounds in a complex matrix. Single compound identification was realized by use of the accurate mass (m/z), the presence of the ^{13}C-isotope and a stable $^{13}C/^{12}C$-ratio of each target ADR and the retention time relative to that of the internal standard (deviation $\leq$ 2.5%), all being investigated from the corresponding reference standard ensuring adequate identification and accurate quantitation. Sensitivity and repeatability were adequate with a sufficient number of data points across every chromatographic peak (> 10). The AGC target was optimized and set to $1x10^5$ ions.

3.2. Method validation

3.2.1. Linearity, LOD, LOQ and confirmatory analysis

Good linearity was obtained for all target compounds with $R^2 \geq 0.993$ and $g \leq 11.56\%$, applying a weighing factor of $1/x^2$ for best fitting (Table 1).

HRMS presents new challenges to the determination of LODs and LOQs which are traditionally estimated by theoretical or empirical calculations based on signal-to-noise ratios [14]. Signal-to-noise ratios obtained by HRMS are mainly of infinite magnitude, resulting in virtually low detection and quantification limits. For this reason, new strategies are required based on more practical criteria to deal with these virtual estimations. In this context, the validation criteria stated in CD 2002/657/EC (food safety), CD 2009/90/EC (water monitoring), VICH 49 and ICH guidelines (general guidelines) for measuring residues were combined and practically confirmed by spiking blank water samples at the LOD level. An additional criterion, i.e. identity confirmation through the presence of the ^{13}C-isotope and the $^{13}C/^{12}C$-ratio of each target ADR at the corresponding theoretical LOD was performed. As such, the presence of every target compound was undoubtedly confirmed by detecting the ^{13}C-isotope with the corresponding ^{12}C-isotope [30]. LOD values ranged from 10 to 50 ng L^{-1} for all analytes. The LOQ value for all analyzed ADRs was 50 ng L^{-1} (Table 1). Overall, these LODs and LOQs are comparable [21] or better [22] than those reported in previous studies using UHPLC-HRMS for one or more ADRs (n $\leq$ 11) from the same antimicrobial class. Furthermore, previous research [2,3] has shown that concentrations below the presented LOD values are of limited environmental risk to algae, sludge bacteria and aquatic microorganisms. In addition, having in mind that the method offers the advantage of accurate quantitation and that concentrations of ADRs in ponds near agricultural activities are expected to be at ng L^{-1} levels, the UHPLC-HRMS method is applicable to the quantitative analysis of pond samples, as shown below.

3.2.2. Apparent recovery, precision and measurement uncertainty

All validation parameters with respect to the apparent recovery, precision and measurement uncertainty are summarized in Table 2.

Table 1
Validation results for linearity from 50 to 500 ng L^{-1} (coefficient of determination (R^2) and goodness-of-fit coefficient (g)), limit of quantification (LOQ) and limit of detection (LOD) of the 46 target antimicrobial drugs included in the UHPLC-Q-Orbitrap™ HRMS method.

Antimicrobial Drugs	R^2	g (%)	LOD (ng L^{-1})	LOQ (ng L^{-1})
Amphenicols				
Chloramphenicol	0.9956	6.83	0.05	0.05
Florphenicol	0.9977	5.44	0.04	0.05
Cephalosporins				
Cefapirin	1.0000	0.32	0.03	0.05
Cefquinome	0.9947	6.33	0.02	0.05
Ceftiofur	0.9983	4.44	0.04	0.05
Diaminopyrimidines				
Trimethoprim	0.9973	5.36	0.004	0.05
Lincosamides				
Lincomycin	0.9960	11.56	0.05	0.05
Macrolides				
Erythromycin A	0.9930	8.63	0.03	0.05
Tilmicosin	0.9993	2.99	0.05	0.05
Tylosin	0.9976	4.60	0.05	0.05
Nitrofurans				
Furaltadon	0.9969	5.69	0.04	0.05
Furazolidon	0.9946	7.61	0.04	0.05
Nifursol	0.9969	10.38	0.04	0.05
Nitrofurantoin	0.9985	4.01	0.04	0.05
Penicillins				
2,5-diketopiperazin[1]	0.9973	5.34	0.03	0.05
Cloxacillin	0.9959	6.50	0.02	0.05
Oxacillin	0.9952	7.14	0.03	0.05
Penicillin G	0.9976	8.20	0.03	0.05
Penicillin V	0.9988	3.93	0.01	0.05
Pleuromutilins				
Tiamulin	0.9969	6.00	0.03	0.05
Quinolones				
Ciprofloxacin	0.9942	7.75	0.03	0.05
Danofloxacin	0.9954	7.79	0.04	0.05
Difloxacin	0.9945	7.54	0.03	0.05
Enrofloxacin	0.9928	8.38	0.03	0.05
Flumequine	0.9995	2.68	0.03	0.05
Marbofloxacin	0.9952	7.48	0.01	0.05
Nalidixin acid	0.9947	7.27	0.02	0.05
Norfloxacin	0.9941	7.80	0.03	0.05
Ofloxacin	0.9935	8.22	0.03	0.05
Oxolinic acid	0.9983	7.62	0.03	0.05
Sarafloxacin	0.9954	6.88	0.03	0.05
Sulfonamides				
Sulfachloropyridazine	0.9956	6.30	0.02	0.05
Sulfadiazine	0.9981	4.51	0.02	0.05
Sulfadimethoxine	0.9934	8.41	0.01	0.05
Sulfadoxine	0.9951	7.18	0.01	0.05
Sulfamethoxazole	0.9975	4.94	0.02	0.05
Sulfamerazine	0.9989	3.47	0.02	0.05
Sulfamethazine	0.9973	5.35	0.01	0.05
Sulfathiazole	0.9984	4.15	0.001	0.05
Tetracyclines				
4-epichlortetracycline	0.9957	5.31	0.03	0.05
4-epioxytetracycline	0.9970	4.16	0.03	0.05
4-epitetracycline	0.9907	8.19	0.05	0.05
Chlortetracycline	0.9943	7.53	0.04	0.05
Doxycycline	0.9997	1.61	0.03	0.05
Oxytetracycline	0.9955	6.90	0.05	0.05
Tetracycline	0.9952	7.18	0.03	0.05

1 = amoxicillin-diketopiperazine-2′5′-dione.

Evaluation of the within-day R_{app} in full-scan mode demonstrated that 84.8% of the ADRs spiked at LOQ level (39 of 46) presented values between 74.2% and 109.1%. Seven ADRs (15.2%) showed R_{app} results between 111.3% and 118.3%. For ADRs spiked at 200 ng L^{-1} and 400 ng L^{-1}, within-day R_{app} ranged between 77.1% and 119.4% and between 64.8% and 119.6%, respectively. The evaluation of the between-day R_{app} in full-scan mode indicated that all 46 ADRs spiked at LOQ level showed values between 90.8% and 119.4%. For ADRs spiked at 200 ng L^{-1} and 400 ng L^{-1}, the between-day R_{app} ranged between 72.1% and 113.6% and between 65.7% and 114.5%, respectively for all

Table 2
Validation results of the within-day and between-day precision, apparent recovery and measurement uncertainty (U) for the 46 antimicrobial drugs included in the UHPLC-Q-Orbitrap™ HRMS method.

Antimicrobial Drugs	Within-Day Precision and Recovery (n = 6)						Between-Day Precision, Recovery and Measurement uncertainty (n = 6 × 3)								
	Theoretical concentration 50 ng L^{-1} (LOQ)		Theoretical concentration 200 ng L^{-1}		Theoretical concentration 400 ng L^{-1}		Theoretical concentration (LOQ)			Theoretical concentration			Theoretical concentration		
	Precision (RSD, %)	Recovery (%)	Precision (RSD, %)	Recovery (%)	Precision (RSD, %)	Recovery (%)	Precision (RSD, %)	Recovery (%)	U (%)	Precision (RSD, %)	Recovery (%)	U (%)	Precision (RSD, %)	Recovery (%)	U (%)
Amphenicols															
Chloramphenicol	24.9	99.5	13.7	88.4	12.2	78.6	36.7	94.6	46.8*	24.5	106.8	47.7*	28.5	101.5	48.3*
Florphenicol	21.8	88.0	22.4	86.9	19.3	77.4	17.0	96.1	37.9	21.6	91.5	41.8	25.7	89.6	48.0
Cephalosporins															
Cefapirin	28.1	89.2	23.6	99.5	28.0	108.4	33.2	103.8	36.6*	19.9	101.0	41.9	21.1	107.1	47.0
Cefquinome	14.4	96.6	8.1	99.9	18.7	66.9	21.5	115.3	47.9	6.9	112.3	14.7	18.7	86.6	33.6
Ceftiofur	21.8	111.3	16.9	113.8	19.8	119.6	30.7	104.7	49.0*	15.8	106.0	35.0	26.2	114.5	42.3*
Diaminopyrimidines															
Trimethoprim	10.0	95.0	5.2	93.5	1.9	93.5	9.3	92.9	18.9	6.3	95.5	12.6	9.9	98.4	20.3
Lincosamides															
Lincomycin	1.6	114.5	25.0	77.2	28.4	92.9	17.6	102.3	43.2	18.2	93.4	34.8	21.9	90.7	41.8
Macrolides															
Erythromycin A	28.0	96.6	23.2	99.8	27.4	86.7	26.1	104.6	47.1*	20.7	110.5	41.3	19.4	94.2	38.5
Tilmicosin	11.2	88.6	10.4	105.9	8.6	102.6	37.8	105.9	42.8*	38.5	114.4	29.1*	18.1	109.4	41.7
Tylosin	14.8	117.4	1.9	94.6	26.5	103.0	14.5	110.2	34.1	12.2	98.1	25.9	21.8	95.8	44.3
Nitrofurans															
Furaltadon	22.0	81.8	28.3	97.7	13.1	78.6	35.5	94.8	39.6*	27.1	94.6	43.5*	13.7	98.7	28.2
Furazolidon	19.3	82.3	9.8	102.4	7.1	90.3	18.7	93.9	44.6	11.9	96.9	26.5	27.2	102.6	37.0*
Nifursol	14.0	113.5	8.0	98.1	9.0	99.0	31.8	119.4	35.8*	19.5	96.1	39.1	14.3	102.3	30.5
Nitrofurantoin	29.4	81.3	14.7	85.5	15.4	93.4	27.8	90.8	43.0*	22.1	99.1	46.6	13.6	100.4	29.2
Penicillins															
2,5-diketopiperazin[a]	15.6	109.1	20.5	93.2	14.3	99.6	18.6	107.4	46.8	24.1	92.3	46.3	25.0	82.1	42.7
Cloxacillin	8.6	114.8	12.6	103.9	12.0	114.0	22.7	112.3	31.9*	32.6	112.9	30.7*	11.5	106.0	26.1
Oxacillin	21.9	118.3	28.6	112.9	11.5	100.7	37.1	118.7	48.9*	26.0	108.7	48.3*	9.6	103.1	20.6
Penicillin G	14.4	74.2	12.3	94.8	22.7	83.5	25.3	114.7	48.8*	15.6	113.6	33.0	15.2	76.9	25.6
Penicillin V	19.3	100.5	22.3	89.0	7.4	80.1	26.0	115.9	48.9*	33.6	87.1	38.8*	13.2	76.3	21.2
Pleuromutilins															
Tiamulin	9.2	97.5	17.1	89.0	10.4	96.6	5.3	98.7	19.6	7.3	93.4	37.3	5.7	94.5	20.5
Quinolones															
Ciprofloxacin	12.4	106.0	11.0	100.8	15.7	109.9	11.4	105.2	25.0	11.8	100.8	24.8	11.6	104.8	25.4
Danofloxacin	7.3	103.6	14.6	102.6	22.3	113.7	10.5	106.1	23.2	15.1	102.4	32.4	17.6	106.6	39.0
Difloxacin	9.9	105.6	10.7	97.9	12.1	108.6	10.7	103.5	23.0	11.8	98.8	24.4	11.5	102.0	24.4
Enrofloxacin	10.1	96.0	10.7	96.2	13.8	114.0	11.6	99.7	24.2	14.5	100.8	30.4	13.5	106.7	30.0
Flumequine	7.8	112.3	15.9	105.0	23.6	109.9	20.4	98.8	48.2	18.2	98.9	37.4	18.3	108.7	41.3
Marbofloxacin	10.5	97.1	4.9	105.0	10.1	111.7	13.8	98.2	28.3	11.4	100.7	23.8	12.3	103.5	26.4
Nalidixin acid	16.2	99.2	7.8	99.4	14.5	109.0	14.3	105.9	31.5	15.6	103.7	35.7	11.8	105.1	25.9
Norfloxacin	12.5	108.9	9.9	100.9	9.5	107.5	12.3	106.2	27.2	11.3	100.3	23.6	10.3	100.7	23.3
Ofloxacin	12.7	105.9	10.4	98.8	8.3	107.2	12.5	100.5	26.1	12.2	97.7	24.9	13.2	101.8	28.5
Oxolinic acid	12.1	84.5	16.4	101.6	19.9	64.8	15.8	90.9	47.2	18.6	94.4	31.8	30.9	73.3	37.1
Sarafloxacin	13.7	107.9	11.0	99.6	9.7	111.8	13.1	108.5	29.5	11.8	99.4	24.4	11.0	102.0	25.5
Sulfonamides															
Sulfachloropyridazine	19.0	106.4	7.9	99.2	5.2	98.1	14.4	100.0	30.0	7.9	95.7	17.0	9.5	95.5	21.6
Sulfadiazine	22.3	87.2	4.4	97.8	8.4	92.3	24.6	92.7	47.4	9.8	95.4	21.0	14.8	96.6	33.0
Sulfadimethoxine	10.0	88.7	8.1	92.9	4.4	96.5	10.9	100.1	26.3	8.6	96.4	18.6	7.1	99.9	16.4
Sulfadoxine	13.8	92.1	6.7	93.0	4.7	99.2	11.4	97.5	23.1	9.8	95.9	22.5	7.6	98.4	17.0
Sulfamethoxazole	14.8	101.6	4.6	103.2	5.5	105.4	10.5	101.5	22.1	6.7	99.7	14.2	7.8	102.2	16.7
Sulfamerazine	19.4	87.7	3.8	99.7	8.5	92.4	13.8	94.3	27.3	6.2	96.6	12.4	7.1	96.0	14.3
Sulfamethazine	11.5	90.9	4.0	91.4	3.7	94.0	9.4	96.7	19.6	6.8	94.0	13.6	4.0	95.5	8.6

(*continued on next page*)

Table 2 (*continued*)

Antimicrobial Drugs	Within-Day Precision and Recovery (n = 6)						Between-Day Precision, Recovery and Measurement uncertainty (n = 6 × 3)					
	Theoretical concentration 50 ng L^{-1} (LOQ)		Theoretical concentration 200 ng L^{-1}		Theoretical concentration 400 ng L^{-1}		Theoretical concentration (LOQ)			Theoretical concentration			Theoretical concentration		
	Precision (RSD, %)	**Recovery (%)**	**Precision (RSD, %)**	**Recovery (%)**	**Precision (RSD, %)**	**Recovery (%)**	**Precision (RSD, %)**	**Recovery (%)**	**U (%)**	**Precision (RSD, %)**	**Recovery (%)**	**U (%)**	**Precision (RSD, %)**	**Recovery (%)**	**U (%)**
Sulfathiazole	18.5	98.2	3.5	99.2	9.9	92.3	15.8	96.0	33.5	7.6	96.5	16.5	9.5	93.9	18.6
Tetracyclines															
4-epichlortetracycline	4.2	107.7	29.7	106.3	15.6	68.7	15.7	93.5	34.0	37.2	72.1	42.7*	32.4	65.7	44.2
4-epioxytetracycline	23.5	82.7	23.0	119.4	13.3	94.6	16.5	92.7	32.0	34.5	112.0	42.5*	16.5	96.3	44.2
4-epitetracycline	6.2	89.5	10.4	97.8	6.1	98.5	23.2	94.5	46.9	14.4	102.4	27.9	19.6	90.0	40.7
Chlortetracycline	6.0	98.6	28.8	77.1	25.9	66.1	29.8	99.1	43.8*	24.8	90.0	49.0*	31.3	67.5	48.1
Doxycycline	14.5	102.3	20.4	101.9	8.3	101.5	23.5	92.6	49.7	36.2	83.9	42.9*	31.4	97.2	28.3*
Oxytetracycline	24.8	99.3	3.7	105.5	9.1	97.7	22.5	90.8	42.6	36.1	117.9	21.0*	32.3	90.0	46.0*
Tetracycline	19.1	78.7	12.7	81.2	20.8	98.0	27.2	93.3	39.0*	14.6	88.8	31.0	29.9	109.1	40.4*

[a] amoxicillin-diketopiperazine-2′5′-dione * = a maximum of 2 outliers was excluded from the calculation. Outliers were determined using SPSS® boxplots.

compounds. Overall, these results are comparable [21,22] or even better [23] (i.e. penicillins) than those reported in previous studies detecting ADRs in fresh water. One explanation for higher recoveries for penicillins could be that samples, in contrast to previous studies, were not acidified before extraction. It has been reported that penicillins are prone to degradation under acidic or basic conditions and acidification before elution is not optimal for this class of antimicrobials [37]. Overall, the lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone co-polymer in this cartridge provided good results for most polar and apolar analytes included in this study, demonstrating its applicability. Furthermore, the within-day and between-day precision RSDs were below 30% and 40% respectively, for all compounds, showing acceptable precision according to the guideline described above [27]. Results of the measurement uncertainty were within accepted limits (i.e. < 50%), according to the European guideline CD 2009/90/EC. These findings illustrate the well-known limitation of multi-residue methodologies, where not the best conditions for all individual target analytes can be achieved but a compromise on final analytical conditions should be made [37].

3.2.3. *Carry-over and specificity*

High specificity proves that the analytical method enables to differentiate between the target analyte and other compounds or interferences. For this UHPLC-HRMS multi-method, no interference peaks were detected in blank samples within a retention time margin of 5% for all target compounds at their accurate mass. All target ADRs were identified based upon their relative retention time, i.e. the ratio of the retention time of the analyte and its internal standard, and accurate mass. The low retention time deviations ($\leq$ 0.05 min) and observed mass deviations ($\leq$ 1 ppm) confirm the excellent instrumental stability of the newly developed UHPLC-HRMS method (Table S1). Finally, no carry-over was detected in any of the samples (MeOH/water) injected directly after every calibrator (50–500 ng L^{-1}). These results are in line with previous studies demonstrating that 100% organic solvent kept during at least 1 min during the elution gradient suffices to avoid carry-over contamination [37].

3.3. *Application to freshwater samples from Flemish ponds*

3.3.1. *Targeted analysis*

Analysis of fresh water samples from 18 Flemish ponds was performed to demonstrate the applicability of the newly developed and validated UHPLC-HRMS method. Overall, 20 ADRs were detected in the different samples. In general, tetracycline antimicrobial drugs were found in 17 out of 18 ponds suggesting their widespread environmental prevalence. Highest concentrations were observed for tetracycline, oxytetracycline, doxycycline and their transformation products (i.e. 4-epitetracycline and 4-epioxytetracycline), with concentrations ranging between 50.7 and 248.2 ng L^{-1} and below the LOQ (< 50 ng L^{-1}). Furthermore, enrofloxacin was detected in three ponds below the LOQ and at 125.0 and 132.8 ng L^{-1}. Penicillin G was detected in eight ponds, in one pond at 64.9 ng L^{-1} and below the LOQ in the others. The presence of tetracyclines and their transformation products, as well as enrofloxacin and penicillin G in the aquatic environment results from surface runoff of animal excreta applied on land, attributed to the regular use of these ADRs in veterinary practices [38]. Oxolinic acid was detected at concentrations ranging between 57.1 and 146.8 ng L^{-1} in 7 out of 18 ponds. The use of oxolinic acid in human medicine is prohibited in several countries including Belgium and replaced by the use of other more effective fluoroquinolones. However, it is still frequently used in veterinary medicine to treat urinary infections produced by *Colibacillus* sp. in livestock. For this reason, oxolinic acid may be present in animal excreta which can eventually end up in the aquatic environment as well [39]. Nine ADRs were found below the LOQ including sulfadiazine, sulfadoxine, sulfamerazine, marbofloxacin, nalidixic acid, tylosin, florphenicol, tiamulin and trimethoprim. Despite penicillins

Table 3
Concentrations in ng L^{-1} of the target antimicrobial drug residues in grab samples taken at 18 different ponds across Flanders, Belgium. Each mixed sample was analyzed in threefold, the mean ± standard deviation is shown.

Pond identification code	Sampling Campaign 2018																	
	BRA 2a	BRA 2b	BRA 3	BRA 4	BRA 6	ZOT 1	ZOT 4	ZOT 5	ZOT 6	ZOT 7	ZOT 8	ZOT 9	ZOT 10	GER 1	GER 2	MAARK1	MAARK2	LIE
Amphenicols																		
Florphenicol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diaminopyrimidines																		
Trimethoprim	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Macrolides																		
Tylosin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	< LOQ	n.d.
Penicillins																		
2,5-Diketopiperazin[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ
Penicillin G	< LOQ	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	< LOQ	n.d.	< LOQ	< LOQ	n.d.	n.d.	n.d.	64.9 ± 2.6	n.d.	< LOQ	< LOQ
Pleuromutilins																		
Tiamulin	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quinolones																		
Enrofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	125.0 ± 28.1	n.d.	n.d.	132.8 ± 23.1
Marbofloxacin	< LOQ	n.d.	< LOQ	< LOQ	< LOQ	< LOQ	n.d.	< LOQ	< LOQ	< LOQ	n.d.	n.d.	n.d.	< LOQ	n.d.	< LOQ	n.d.	n.d.
Nalidixic acid	n.d.	n.d.	n.d.	< LOQ	< LOQ	n.d.	< LOQ	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	< LOQ
Oxolinic acid	54.4 ± 6.2	n.d.	72.9 ± 24.6	n.d.	70.4 ± 24.3	60.23 ± 3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	146.8 ± 45.8	57.1 ± 2.8	n.d.	n.d.	n.d.	83.8 ± 29.4
Sulfonamides																		
Sulfadiazine	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfadoxine	< LOQ	n.d.	n.d.	n.d.	< LOQ	< LOQ	n.d.	< LOQ	< LOQ	n.d.	< LOQ	n.d.	< LOQ	< LOQ	n.d.	n.d.	n.d.	< LOQ
Sulfamerazine	n.d.	n.d.	n.d.	< LOQ	n.d.	< LOQ	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	< LOQ
Tetracyclines																		
4-epichlortetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	78.9 ± 5.5	n.d.
4-epioxytetracycline	n.d.	n.d.	n.d.	n.d.	52.3 ± 6.9	n.d.	n.d.	n.d.	50.7 ± 3.1	55.0 ± 6.9	55.3 ± 6.9	n.d.	78.5 ± 6.4	47.4 ± 2.6	n.d.	n.d.	n.d.	n.d.
4-epitetracycline	n.d.	n.d.	76.5 ± 16.3	n.d.	88.3 ± 6.8	n.d.	n.d.	51.9 ± 5.3	83.0 ± 37.1	65.5 ± 22.8	75.8 ± 10.1	n.d.	173.6 ± 66.5	54.0 ± 3.7	n.d.	n.d.	n.d.	145.2 ± 69.6
Chlortetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	60.0 ± 6.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Doxycycline	n.d.	55.5 ± 4.1	111.1 ± 35.4	n.d.	80.2 ± 25.7	n.d.	54.0 ± 7.0	68.0 ± 13.0	58.1 ± 4.2	89.1 ± 13.8	n.d.	n.d.	141.3 ± 57.7	63.9 ± 7.5	n.d.	80.1 ± 26.1	72.0 ± 29.1	125.0 ± 33.9
Oxytetracycline	49.6 ± 4.0	n.d.	78.7 ± 9.9	n.d.	69.0 ± 21.1	n.d.	n.d.	n.d.	56.6 ± 3.1	97.5 ± 1.4	83.7 ± 37.6	48.0 ± 6.5	229.6 ± 65.8	61.0 ± 15.0	83.9 ± 31.8	52.5 ± 3.9	50.7 ± 5.5	110.4 ± 32.8
Tetracycline	n.d.	n.d.	111.6 ± 10.9	n.d.	152.0 ± 53.3	65.7 ± 8.5	74.5 ± 13.6	60.0 ± 2.1	85.1 ± 38.8	107.0 ± 44.8	189.3 ± 84.0	57.3 ± 2.7	246.1 ± 81.6	68.8 ± 17.6	106.7 ± 50.4	60.1 ± 6.5	73.0 ± 31.1	248.2 ± 76.7

[a] amoxicillin-diketopiperazine-2′5′-dione. n.d. = not detected < LOQ = below the method quantification limit (50 ng L^{-1}).

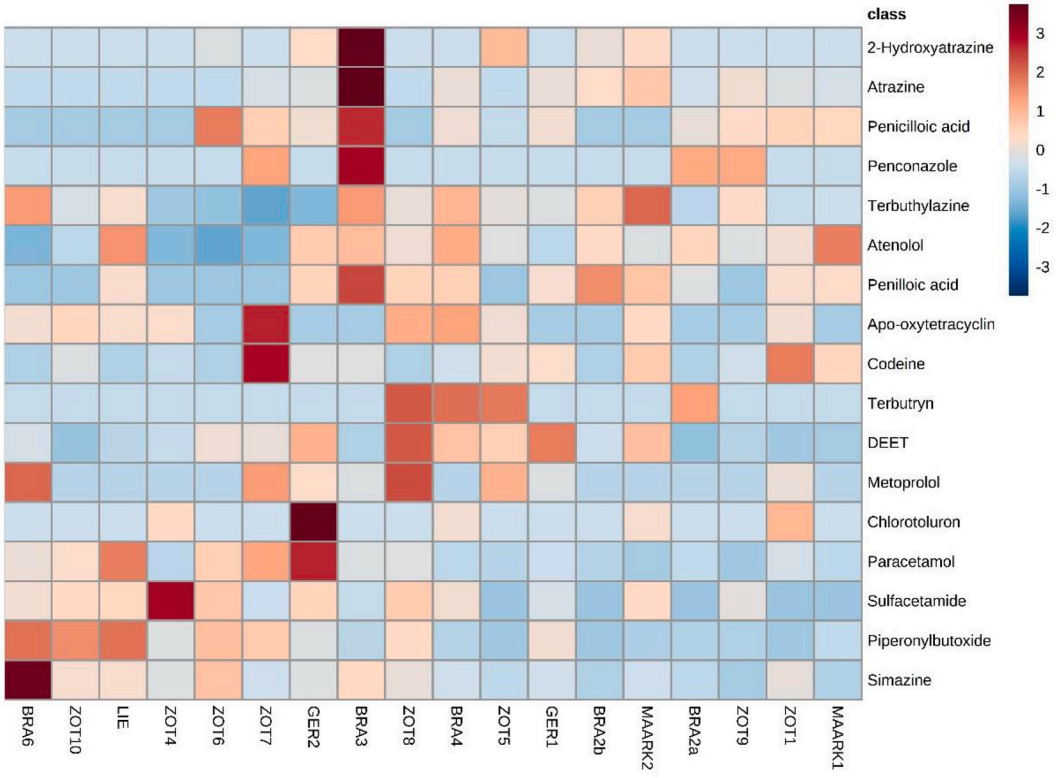

Fig. 3. Heatmap of the suspect screening and confirmatory analysis using our in-house database on additional pharmaceuticals (n = 107) and pesticides (n = 83), presenting respectively 8 and 9 compounds. Each colored cell on the map corresponds to the relative peak area for a particular compound (row) in a particular sample (column). Data was sum normalized and autoscaled. Relative peak area levels are indicated on the color scale, with numbers indicating the fold difference from the overall average. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

being the most consumed group of antimicrobials, detected concentrations remain relatively low in environmental samples. We expect this to be the result of degradation due to the chemically unstable β-lactam ring, which is highly sensitive to heat, pH and β-lactamase enzymes. Interestingly, a possible metabolite of the highly consumed amoxicillin used in veterinary and human medicine (i.e. amoxicillin-diketopiperazine-2′,5′-dione) was found in only one pond at trace level. However, further degradation of amoxicillin-diketopiperazine-2′5′-dione and the fact that studies have shown that this compound is an intermediate rather than stable-end product, could account for the fact that the amoxicillin-metabolite is only rarely detected [40]. Results of the targeted analysis are presented in Table 3.

3.3.2. *Suspect screening and confirmatory analysis*

Suspect analysis was performed by screening the full-spectrum acquisitions against our in-house database of additional pharmaceuticals (n = 105) (Table S5) and pesticides (n = 83) (Table S6), resulting in 4370 and 1927 candidates, respectively. As ESI generally produces molecular ions $[M+H]^+$ or $[M-H]^-$, initial suspect screening was performed using these masses. Additionally, other adducts (i.e. $[M+Na]^+$, $[M+CH_3OH+H]^+$, $[M+FA-H]^-$) were included as well. Based on accurate mass (5 ppm), the presence of the ^{13}C-isotope and a stable C^{12}/C^{13}-ratio, 14 pharmaceuticals including 3 antimicrobial degradation products and 11 pesticides were tentatively identified. Most intense (grey) and second most intense adduct ions (light grey) of the compounds found during suspect screening are denoted in Table S7. To obtain the highest level of confirmation (i.e. level 1), reference standards were analyzed for every suspect compound, except for apo-oxytetracycline, amoxicillin penicilloic acid, amoxicillin penilloic acid and codeine, for which no reference standards were available at the time of the analysis. For 13 compounds, proposed structures were confirmed through matching accurate mass (deviation limit < 3 ppm), RT time (deviation ≤ 2.5%) and the presence of the ^{13}C-isotope, resulting in a level 1 confirmed structure, as described by Schymanski et al. (2014) [34]. For apo-oxytetracycline, amoxicillin penicilloic acid, amoxicillin penilloic acid and codeine, a level 2a probable structure was proposed by the presence of the C_{13}-isotope and matching literature spectrum data i.e. accurate mass and retention time resulting from studies using an UPLC-HRMS method including a C18 column, similar mobile phases and mass spectrometric parameters [40,41]. A total of 17 identified structures are presented in Fig. 3. Results are not surprising, as it is known that the epimeric form of oxytetracycline (found in 6 out of 18 ponds during targeted screening) and the highly used amoxicillin, degrade quickly in aquatic matrices [40]. Furthermore, certain pharmaceuticals such as paracetamol (i.e. analgesic and antipyretic agent) and metoprolol (i.e. beta-blocker) as well as popular pesticides such as terbuthylazine (i.e. triazine herbicide) and chlorpyrifos (i.e. organophosphorus insecticide) are commonly found environmental contaminants [8,42].

4. Conclusion

A novel multi-residue SPE-UHPLC-Q-Orbitrap™ HRMS method was successfully developed and validated for the simultaneous detection and quantification of 46 antimicrobial drug residues from 11 different antimicrobial classes in fresh pond water. Validation results demonstrated good linearity as well as acceptable within-run and between-run apparent recoveries and satisfactory precision according to the guidelines discussed above. The empirical LOD values in fresh water ranged from 1 to 50 ng L^{-1} for all analytes. The LOQ value for all analyzed ADRs was 50 ng L^{-1}. These low values are comparable to those reported in previous studies using UHPLC-HRMS for one or more ADRs (n ≤ 11) from the same antimicrobial class and are essential for the environmental application of this novel method. Moreover, the presented

analytical method is the first to detect as well as quantify over forty ADRs from different antimicrobial classes (log P ranging from −4.05 to 4.38), including transformation products and one primary metabolite, at environmental concentrations in pond water. Furthermore, by making use of HRMS rather than triple quadrupole mass spectrometry, this method provides the possibility to detect compounds without preselection using untargeted or suspect screening, resulting in a highly suitable multi-residue method for environmental screening of aquatic contaminants in pond water. Additionally, our newly developed method may provide insights about the role of pollution in host-pathogen dynamics of chytridiomycosis in amphibians. As such, it may contribute to highly demanded mitigating strategies, preventing further loss of biodiversity worldwide.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

T. Goessens: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Writing - original draft. **S. Huysman:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **N. De Troyer:** Investigation, Writing - review & editing. **A. Deknock:** Investigation, Writing - review & editing. **P. Goethals:** Funding acquisition, Resources, Writing - review & editing. **L. Lens:** Funding acquisition, Writing - review & editing. **L. Vanhaecke:** Conceptualization, Resources, Writing - review & editing. **S. Croubels:** Funding acquisition, Writing - review & editing, Conceptualization.

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Appendix A. Supplementary data

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